

Does size matter for horny beetles? A geometric morphometric analysis of interspecific and intersexual size and shape variation in *Colophon haughtoni* Barnard, 1929, and *C. kawaii* Mizukami, 1997 (Coleoptera: Lucanidae).

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Title: Does size matter for horny beetles? A geometric morphometric analysis of interspecific and intersexual size and shape variation in *Colophon haughtoni* Barnard, 1929, and *C. kawaii* Mizukami, 1997 (Coleoptera: Lucanidae).

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Abstract

Colophon is an understudied, rare and endangered stag beetle genus with all species endemic to isolated mountain peaks in South Africa's Western Cape. Geometric morphometrics was used to analyse intersexual and interspecific variation of size and shape in the mandibles, heads, pronota and elytra of two sympatric species: *C. haughtoni* and *C. kawaii*. All measured structures showed significant sexual dimorphism, which may result from male-male competition for females. Female mandibles were too small and featureless for analysis, but male *Colophon* beetles possess large, ornate mandibles for fighting. Males had significantly larger heads and pronota that demonstrated shape changes which may relate to resource diversion to the mandibles and their supporting structures. Females are indistinguishable across species, but males were accurately identified using mandibles, heads and pronota. Male *C. kawaii* were significantly larger than *C. haughtoni* for all structures. These results support the species status of *C. kawaii*, which is currently in doubt due to its hybridisation with *C. haughtoni*. We also demonstrate the value of geometric morphometrics as a tool which may aid *Colophon* conservation by providing biological and phylogenetic insights and enabling species identification.

Keywords: *Colophon* – Lucanidae – geometric morphometrics – morphology – species identification – sexual dimorphism.

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49

Introduction

Colophon Gray, 1832, is a genus of endangered montane stag beetles (subfamily Lucaninae Latreille, 1804 (Kim and Farrell 2015)) endemic to the Western Cape Province of South Africa (Fig. 1) (Geertsema and Owen 2007; Switala et al. 2015). The first species was described by Gray (1832) in 1832, but over 180 years later we still know very little about these understudied beetles

Colophon biology

The genus *Colophon* comprises 17 known species, each endemic to a particular mountain peak or range. All species are slow-moving and flightless, with adult activity spanning October to March (Barnard 1929; Endrödy-Younga 1986; Geertsema and Owen 2007). They inhabit montane fynbos habitats and are closely associated with *Restionaceae* bushes (Barnard 1929; Brinck 1956). Little else is known about the ecology, demography, physiology or behaviour of the genus (Geertsema and Owen 2007; Roets et al. 2013).

Colophon conservation

Colophon beetles are a research and conservation priority. Locally endemic species are especially vulnerable to extinction (Van Dyke 2008; Pizzo et al. 2011) and the restriction of *Colophon* species to the Western Cape mountain peaks makes them particularly vulnerable to global warming (Parmesan et al. 1999; Switala et al. 2015). Construction projects are damaging and reducing the already limited habitats of some populations (Geertsema and Owen 2007) and all *Colophon* species are threatened by illegal collection, with single specimens being sold for up to US\$15,000 on the black market (Beeton 1997; Melisch and Schütz 2000). *Colophon*

is an important flagship taxon for campaigns against illegal insect trade (New 2009) and all species are listed on CITES Appendix III (CITES 2015). The genus was added to the South African ToPS list in 2007 and species are also categorized from vulnerable to critically endangered on the IUCN Red List (IUCN 2015). However, these assessments need updating (IUCN 2015) and the effective management and conservation of *Colophon* is impaired by the paucity of knowledge on their natural history (New 2009; Roets et al. 2013).

Morphology

This study is the first attempt to statistically examine morphological variation in *Colophon* stag beetles. As morphology is determined by both genotype and phenotype it can provide insights into the phylogeny and ecology of *Colophon* beetles and the selective pressures driving their evolution (Losos and Miles 1994). A morphological study can also aid the development of a reliable and accurate identification method for this genus, which is fundamental to studying *Colophon* biology and ultimately to conserving the genus.

Traditionally, shape analysis relied on qualitative descriptions and linear measurements. These were often inadequate for describing the complexity of many organisms and the slight (but significant) variations between them. Modern geometric morphometrics provide a fast, cheap and accurate method for the detection, quantification and visualisation of subtle shape changes between organisms even at the intraspecific level (Bookstein 1997; Alibert et al. 2001; Adams et al. 2004; 2013).

***Colophon* morpho-taxonomy**

Colophon morphology was first investigated by Endrödy-Younga (1986), who used morphological characters to create a dichotomous key for the genus and hypothesize phylogenetic relationships between species. Switala et al. (2014) have recently confirmed these predictions using molecular techniques highlighting the value of morphology as a reliable and powerful taxonomic tool. The phylogenetic placement of one species, *C. kawaii*, remains unsolved (Switala et al. 2014). This species was undiscovered during Endrödy-Younga's study and is consequently not included in the identification key for the genus. Furthermore, Switala *et al.* analysed only two *kawaii* specimens, which either grouped as sister to *C. cameroni* Barnard, 1929, or nested within *C. haughtoni*. The species status of *kawaii* is further confounded by molecular evidence for its hybridisation with *haughtoni*, with which it occurs sympatrically in the Hex River Mountains (Switala 2013; Switala et al. 2014). Geometric morphometrics have been successfully used to distinguish honeybee subspecies (*Apis mellifera* Linnaeus, 1761; Hymenoptera: Apidae) (Tofilski 2008) and to identify cryptic *Nebriola* ground beetle species (Coleoptera: Carabidae) (Roggero et al. 2013), and similar methods could shed light on *Colophon* systematics.

Sexual dimorphism in *Colophon*

Male *Colophon* possess large mandibles which are highly diverse and species-specific in shape. By contrast, female mandibles are small, non-diagnostic structures (Endrödy-Younga 1986). Such sexual dimorphism may result from male-male competition for females; sex ratios in this genus appear to be male-biased for all species and male *Colophon* have been observed to attack each other using their mandibles (Geertsema and Owen 2007; T.E., pers. obs.). Male beetle weaponry is often positively allometric with body size (Petrie 1988; Kawano 1997; Kodric-Brown

et al. 2006) and larger weapons and/ or body sizes improve a males fighting success (Moczek & Emlen 2000; Moczek 2006). This favours sexual selection for increasingly large, weaponized males and is a likely driver of sexual dimorphism in many Coleopteran species including *Colophon* (Bonduriansky 2007; Painting and Holwell 2013). Identifying and quantifying sexual dimorphism in *Colophon* is the first step towards a full understanding of the selective pressures driving their evolution (Emlen 1997; Moczek 2006).

Aims and hypotheses

This study will focus on two sympatric species: *C. haughtoni* and *C. kawaii*. The research need for these species is highlighted by the IUCN Red List (IUCN 2015); the conservation status of *C. haughtoni* is endangered but requires updating, whilst *C. kawaii* is not even listed. Switala (2013) suggests that the latter species is critically endangered. Geometric morphometrics will be employed to quantify, visualise and test the statistical significance of interspecific and intraspecific size and shape variation in *Colophon* using four structures: the mandible, head, pronotum and elytron. Two hypotheses will be tested: (1) all of the above structures demonstrate sexual dimorphism in the species *C. haughtoni* and *C. kawaii*, and (2) all listed structures differ significantly in size and shape between male *C. haughtoni* and male *C. kawaii* beetles. Two additional aims of this study are (i) to provide support for the hypothesis that *C. kawaii* is a distinct species by testing the degree of morphological separation between male *C. haughtoni* and *C. kawaii* and (ii) evaluate the accuracy and value of geometric morphometrics as a tool for identifying *Colophon* species.

Methods

Data collection

Data were collected near the peak of Matroosberg Mountain by searching under shrubs for dead beetle fragments (04/Nov/2014- 10/Jan/2015; Location: S33°23' E19°40' 2231m; CapeNature Permit 0056-AAA007-00129). Sexes were distinguished using the mandibles, but females could not be identified to species level due to their extreme similarity and the consequent lack of any taxonomic key. Male *Colophon* beetles were identified using traditional qualitative characters based on Endrödy-Younga's (1986) dichotomous key. As previously mentioned this key does not include *C. kawaii*. However Endrödy-Younga describes how *C. haughtoni* can be distinguished from other *Colophon* species by the presence of “a flattened shiny surface” which connects the apex of the mandible and the dorsal process (see Fig. 1 for anatomical characters). This structure was found to be absent in *C. kawaii* (Figs. 1, 2, and so can be used as a basic, qualitative character to differentiate between these two species.

Fragmentary specimens were organised into the following groups: male *haughtoni*, male *kawaii*, females and “unidentified” *Colophon* individuals whose species and sex could not be determined. Complete specimens were dissected into the head, prothorax and abdomen. Pronota were separated from the prosterna and associated legs, and the elytra were separated from the abdomens and legs. This flattened out the pronota and elytra and avoided errors associated with placing 2D landmarks on a 3D object (Cardini 2014). Segments were photographed by placing them on a stage with graph paper underneath. All segments were photographed dorsally and heads were also photographed ventrally to view the mandibles. A camera (Panasonic Lumix DMC-FT2 with a 28mm lens) was mounted above with the lens at a distance of 350mm from the stage. The camera was set at an angle parallel to the stage using

a spirit level. Fragments were placed in the centre of the image, which preliminary tests showed was free from distortion. Four datasets were created: mandible (n *C. haughtoni* = 39; n *C. kawaii* = 12); head (n *C. haughtoni* = 57; n *C. kawaii* = 13; n female = 19); pronotum (n *C. haughtoni* = 32; n *C. kawaii* = 11; n female = 26; n unidentified = 104) and elytron (n *C. haughtoni* = 20; n *C. kawaii* = 33; n female = 10; n unidentified = 113).

Geometric morphometrics

Geometric morphometrics is the analysis of shape using geometric Cartesian coordinates instead of traditional qualitative variables or linear measurements (Zelditch et al. 2004). In geometric morphometrics the shape of organisms is described using a series of landmarks which are defined as discrete anatomical loci that are homologous across all specimens in the study (Zelditch et al. 2004). Shape information can be extracted from landmark coordinates and subjected to multivariate statistical analysis in order to answer a wide range of biological questions (Adams et al. 2004; 2013).

For each dataset in this study landmarks were digitized on photographs using tpsDig2 ver. 2.17 (Rohlf 2013; 2015; Fig. 2). Only the left side of structures were digitized to avoid bias caused by bilateral asymmetry (Marrone et al. 2014). Mandibles were analysed for males only as female mandibles were too small to reliably identify homologous landmarks. Procrustes superimposition was performed in MorphoJ ver. 1.06b (Klingenberg 2011) to scale, translate and rotate the landmark coordinates and produce Procrustes coordinates which contain information on shape only.

Shape variation and visualisation

A covariance matrix of the shape coordinates was generated in MorphoJ and a principal component analysis (PCA) performed on the matrix (preliminarily excluding unidentified beetles). Shape variation was visualised along each PC axis using thin-plate spline deformation grids produced in tpsRelw ver. 1.53 (Rohlf 2013; 2015).

Size and allometry

The size differences between groups (female, *C. haughtoni*, *C. kawaii*) were tested for statistical significance using IBM SPSS Statistics 22. Each dataset was analysed separately. The natural log transformed centroid size (LnCS) was used as a measure of size for each individual. Centroid size is the square root of the summed squared distances between all landmarks and their centre of gravity or centroid (Bookstein 1997). A Shapiro-Wilk test showed that all LnCS distributions were normal ($p > 0.05$). Levene's test assessed the datasets for homogeneity of variances. The mandible and elytron datasets were then tested for differences between species and sexes using a one-way ANOVA followed by Tukey post-hoc tests to determine which groups differed significantly in LnCS. The head and pronotum datasets showed significant heterogeneity of variances ($p < 0.05$), so Welch's ANOVAs and Games-Howell post-hoc tests were applied instead.

A multivariate regression in tpsRegr ver. 1.41 (Rohlf 2013; 2015) tested for allometric effects by regressing partial warps (dependent variable) on LnCS (independent variable). Partial warps are shape variables calculated in tpsRegr from the raw landmark data. "Unidentified" beetles were again preliminarily excluded and each dataset was analysed separately. Deformation grids for the smallest, middle sized and largest individuals were constructed in tpsRegr to visualise the shape deformations associated with size changes.

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219 **Group classification and differentiation**

220 The software PAST (Hammer et al. 2001) was used to test the significance of shape
221 differences between male *C. haughtoni*, male *C. kawaii* and females (unidentified
222 beetles were excluded). One-way non-parametric MANOVAs based on the
223 Euclidean distance measure were performed on the PC scores for the mandible,
224 head, pronotum and elytron datasets. Significance levels were calculated by
225 permutation of beetle group membership with 9,999 permutations. Pairwise non-
226 parametric MANOVAs between all pairs of groups were implemented as post-hoc
227 tests.

228 Discriminant function analyses (DFA) using cross-validation methods were
229 performed in IBM SPSS Statistics 22 to statistically test the ability of size and shape
230 variables to predict which beetle group a specimen belonged to. Beetle category
231 (male *C. haughtoni*, male *C. kawaii* and female) was used as the factor and LnCS
232 and principal component (PC) scores were used as independent variables.
233 Unidentified individuals were included and categorised as unknown. Procrustes
234 Coordinates and LnCS were also used as independent variables but were less
235 accurate and so were discarded (see also Meloro 2011 and Meloro et al. 2015).
236 Stepwise methods were employed and a variable was entered into the model if the
237 probability of its *F*-value was greater than 0.05 and removed if the probability value
238 was lower than 0.10.

239 **Results**

240 **Shape variation and visualisation**

The mandible PCA shows two distinct clusters for *C. haughtoni* and *C. kawaii* when PC1 is plotted against PC2 (Fig. 3a). Together PC1 and PC2 explain 83.63% of total variance in the sample. *C. haughtoni* specimens are grouped at the extreme right of PC1, and are shown in the thin-plate spline to be more elongated with a longer ventral process (see Fig. 1 for anatomical traits). *C. kawaii* mandibles group at the extreme negative of PC1, being characterised by a squatter overall shape with a relatively short ventral process and a pronounced indent between the apex and dorsal process of the mandible.

PC1 and PC2 explain 77.89% of variance in the head PCA (Fig. 3b). This plot produces a total separation between male and female beetles along PC1, which mainly describes the relative length of the head. The thin-plate splines show females to have a protruding “forehead” or frons whilst males have a larger pre-orbital area at the front corner of the head (represented by the top left landmark in Fig. 3b). Although heterospecific males show substantial overlap in shape space, *kawaii* specimens are positioned more towards the positive end of the PC2 axis which describes a shorter, broader head shape than for *haughtoni* males.

Pronotum shape also produces a good separation of sexes (Fig. 3c). PC1 and PC2 explain 80.77% of the total variance in this structure and display differences between sexes and heterospecific males respectively. Females are distributed at the positive end of PC1 and their pronota are slightly squatter and have shorter, narrower anterior regions compared to males. *C. kawaii* males have more protrusive shoulders than females and *haughtoni* males.

PC1 and PC2 explain 86.56% of variation in the elytra (Fig. 3d). This plot does not show any separation between groups but males generally have lower PC1 scores

than females. Thin-plate splines show that PC1 describes the relative elytron length and that male beetles have relatively shorter elytra compared to females. Changes on PC2 relates to a relative enlargement (negative scores) or shortening (positive scores) of the anterior edge of the elytra (Fig. 3d).

Size and allometry

Females consistently have the smallest LnCS for each body structure followed by male *C. haughtoni* then male *C. kawaii* (Figs. 4a-d). ANOVAs and post-hoc tests showed highly significant differences between all groups for the LnCS of every body structure (Table 1; all post-hoc tests: $p \leq 0.001$). Regression analyses showed a significant correlation between size and shape for all body structures indicating strong allometry in all structures (Table 1). Allometry was visualised using deformation grids which depicted the shapes of the smallest and largest individuals and their deformation from the mean shape (Figs. 4a-d).

Allometry explains 15.8% of variation observed in the mandible dataset (Table 1) and deformation grids indicate a strong shape deformation from small to large mandible sizes (Fig. 4a). The smallest individual, represented by a *C. haughtoni* specimen, shows a contraction of the dorsal process compared to the mean shape for the sample. The largest specimen, a *C. kawaii*, shows an expansion of this region compared to the mean and a more pronounced indent between the dorsal process and the mandibular apex. The ventral process and apex are relatively shorter in the largest beetle.

Allometry accounts for 41.96% of the total shape variation in *Colophon* heads (Table 1). Smaller individuals are characterized by more elongated, convex heads, as represented by the two landmarks on the right side of the deformation grids in Fig.

4b. Larger specimens have comparatively shorter heads, smaller eyes and larger genal and pre-orbital regions.

Allometry explains 42.68% of pronotal shape variation (Table 1). Deformation grids demonstrate that the smallest pronota are wide at the base but become increasingly narrower and shorter towards anterior portions (Fig. 4c). Larger pronota show the opposite trend of having enlarged anterior regions and contracted posterior regions.

Allometry explains 20.2% of variation observed in the elytron dataset. The smallest elytron has a smaller and more posteriorly placed scutellum compared to the mean, whilst the largest elytron depicts a larger, longer scutellum which extends further anteriorly compared to the mean (represented by the bottom right landmarks in Fig. 4d).

Group differentiation and classification

Non-parametric MANOVAs showed that morphometric differences between male *C. haughtoni*, male *C. kawaii* and female *Colophon* were significant for all measured structures ($p \leq 0.02$; Table 2). DFAs indicated that size and shape data from the mandibles, heads and pronota could be used to accurately predict the sex and species status of *C. haughtoni* and *C. kawaii* (Table 3). Data from the elytra could be used to accurately classify male *C. kawaii* and females, but not male *C. haughtoni* (Table 3).

The DFA for the mandible dataset selected LnCS, PC1, PC2, PC3 and PC4 (in order of decreasing loading - see Table 3a) to discriminate beetle groups. One significant discriminant function (DF) was extracted to distinguish between groups (Wilks $\lambda =$

0.091, $\chi^2(5) = 111.62$, $p < 0.0001$). Following cross-validation, the percentage of correct classifications was 100% for both *C. haughtoni* and *C. kawaii*.

Two significant functions were derived for the head DFA (DF1: 93.6% variance, Wilks $\lambda = 0.041$, $\chi^2(10) = 267.951$, $p < 0.0001$; DF2: 6.4% variance, Wilks $\lambda = 0.544$, $\chi^2(4) = 51.196$, $p < 0.0001$). PC's 1, 2, 4, 5 and LnCS maximally differentiated between groups (Table 3b). All females, 82.5% of *C. haughtoni* and 84.6% of *C. kawaii* specimens were correctly classified following cross-validation.

The Pronotum DFA produced two significant DFs (DF1: 91.9% variance, Wilks $\lambda = 0.108$, $\chi^2(8) = 143.711$, $p < 0.0001$; DF2: 8.1% variance, Wilks $\lambda = 0.682$, $\chi^2(3) = 24.733$, $p < 0.0001$) loaded on LnCS, PC1, PC2 and PC8 (Table 3C). The percentage of correct classifications were high for all cross-validated groups (females: 96.2%; *C. haughtoni*: 90.6%; *C. kawaii*: 72.7%).

Although beetle groups could not be separated by plotting elytron PC1 values against PC2 values, two significant discriminant functions were selected for in the DFA (DF1: 95.4% variance, Wilks $\lambda = 0.264$, $\chi^2(6) = 78.683$, $p < 0.0001$; DF2: 4.6% variance, Wilks $\lambda = 0.896$, $\chi^2(2) = 6.458$, $p < 0.0001$). LnCS, PC1 and PC2 were the variables which most separated the groups (Table 3d). Females (85%) and *C. kawaii* (80%) showed high percentages of correct classifications, whilst *C. haughtoni* was correctly classified only 66.7% of the time after cross-validation.

Discussion

This study has made the first exploration of *Colophon* morphology using geometric morphometrics. *C. kawaii* is found at only one location where it occurs sympatrically with *C. haughtoni* (Switala 2013), but there is currently no standard method for

discriminating between these species. We identified a structure (a flattened shiny surface connecting the mandible apex with the dorsal process (Fig. 1)) which is present in *C. haughtoni* but absent in *C. kawaii* and so can be used as a basic qualitative character for distinguishing these species. However, traditional qualitative morphometric characters cannot be subjected to statistical analysis or demonstrate the significance of morphological variations in the genus (Bookstein 1997; Alibert et al. 2001; Adams et al. 2004; 2013). Modern geometric morphometric methods provide a new, analytical perspective which advance the taxonomy of this genus, enable the visualisation of previously undetected shape changes and allow us to answer a range of biological questions regarding *C. haughtoni* and *C. kawaii*.

We found that sexual dimorphism is significant in the heads, pronota and elytra of these species (Hypothesis 1). The morphology of the mandibles, heads, pronota and elytra also differs significantly between male *C. haughtoni* and male *C. kawaii* (Hypothesis 2). This result fulfils our first aim by giving support to the hypothesis that *C. kawaii* is a distinct species. We were able to accurately distinguish sexes and males of different species, suggesting geometric morphometrics are a powerful tool for the classification of *C. haughtoni* and *C. kawaii* (Aim (ii)).

1. Sexual dimorphism

Some studies suggest natural selection, such as divergence in feeding niches, leads to shape divergence between sexes (Temeles and Roberts 1993; Temeles et al. 2000). However, unambiguous examples of ecologically driven sexual dimorphism are rare and most studies suggest sexual selection is the primary cause (Shine 1989). Observations of sexual dimorphism in this study will therefore only be discussed in relation to sexual selection.

Sexual dimorphism is evident in the mandibles of *Colophon* but geometric morphometrics also revealed significant size and shape differences in the head, pronotum and elytron that were not previously apparent (Table 2). Deformation grids allowed the visualisation of subtle shape changes which may have important biological functions. Regression showed that shape variation due to allometry was significant for all structures (Table 1) and females had consistently smaller LnCS values than males (Figs. 4a-d). This may be due to sexual selection for increased overall size in males, which is associated with positive allometric mandible growth and increases their success in male-male competition (Petrie 1988; Kawano 1997; Kodric-Brown et al, 2006).

1.1. Head

Female heads were significantly smaller than in males but were relatively longer with larger eye diameters and a more protrusive frons. Males had a more concave frons, a larger pre-orbital area and larger genae (Fig. 3a). Sexual dimorphism in *Colophon* heads may be related to mandibular form and function. Goyens et al. (2014) found that longer mandibles and stronger bite forces in male *Cyclommatus metallifer* stag beetles (Boisduval, 1835; Coleoptera: Lucanidae) are compensated for by longer input levers and larger closer muscles. These structures in turn require larger heads with broader anterior portions compared to females. A similar mechanism could explain the large size and pre-orbital region of male *Colophon* heads.

Male *Colophon* also had smaller eye diameters and wider genae than females (Fig. 3a). Okada and Miyatake (2009) observed similar trends in large-mandibled *Gnatocerus cornutus* Fabricius, 1798, beetles (Coleoptera: Tenebrionidae) compared to small-mandibled males. They suggest that larger genae may develop in

compensation for enlarged mandibles or may be used for display in male-male competition (Okada and Miyatake 2009). Emlen (2001) demonstrated that excessive horn growth in *Onthophagus* Latreille, 1802, dung beetles can divert resources from nearby structures such as eyes and antennae and a similar trade-off could underlie the relatively smaller eyes of male *Colophon* beetles compared to females.

Male-male competition in *Colophon* beetles may produce sexual selection for increasing mandible size and power in males leading to corresponding sexual dimorphism in head morphometry.

1. 2. Pronotum

Visual examination suggested pronotal morphology was homogenous across sexes but our analyses revealed otherwise. Size was a prominent distinguishing factor between the sexes and females had significantly smaller pronota than males of both species (Table 3, Fig. 4c). Hlavac (1969) and Okada and Miyatake (2009) suggest that larger prothoraces in male beetles may contain a greater muscle mass and energy store and so may evolve to compensate for their large mandibles and the forces produced during combat (Tomkins et al. 2005). This may also explain the expansion of male *Colophon* pronota towards the head region, where most support is needed (Fig. 3c).

Deformation grids also revealed that female pronota are generally squatter than in males and although they are smaller anteriorly they are wider towards the abdomen (Fig. 3c). In contrast to male beetles, females may divert their resources posteriorly to improve their reproductive success. Preziosi et al. (1996) and Adams and Funk (1997) hypothesize that larger abdomens increase the egg carrying capacity and consequently the fecundity of female insects. Fairn et al. (2007) found that the

pronota of female *Dineutus nigrior* Roberts, 1895, beetles (Coleoptera: Gyrinidae) were shorter compared to males and suggested this was in compensation for longer elytra and increased egg storage. Very few studies have explored pronotal sexual dimorphism in beetles but the distinctive pronotal morphology of male and female *Colophon* suggests that this structure may be influenced by sexual selection and so may give insight into the reproductive biology of *C. haughtoni* and *C. kawaii*.

1.3. Elytron

Although elytral shape was significantly different between all beetle groups (Table 2), considerable overlap between sexes was observed in the elytron PCA plots (Fig. 3d). This suggests that sexes are morphologically more similar in their elytra compared to other structures. Size was the most discriminating factor between males and females (Table 3D), and allometric effects were primarily associated with an increase in scutellum size from small females to large males. Females tended towards a relatively longer elytron than males (Fig. 3a) and as previously discussed this may result from selection for increased egg carrying capacity (Preziosi et al. 1996; Adams and Funk 1997). This hypothesis has been used to explain relatively wider abdomens observed in female Chilean Magnificent Beetles (*Ceroglossus chilensis* Eschscholtz, 1829) (Benitez et al. 2011) and the longer abdomens in female *Neochlamisus bebbianae* Brown, 1943, leaf beetles (Adams and Funk 1997). The observed dimorphism in *Colophon* may equally be caused by selection for shorter elytra in males. Kawano (1997) found that elytral size was negatively allometric to body length in Lucanid males and Okada and Miyatake (2009) observed that increased horn growth in male *G. cornutus* beetles was correlated with reduced elytral length. This suggests that resources are diverted anteriorly to structures more important for competition in male beetles (Okada and Miyatake 2009). Our results

indicate gender specific trends in elytral morphology but the detection and visualisation of shape changes was probably hindered by the lack of homologous landmarks on this structure. Further studies should be conducted using semi-landmark methods (Zelditch et al. 2004; Van Bocxlaer and Schultheiß 2010).

Most studies focus on sexual dimorphism in beetle weaponry and little information is available on other body structures. However, gender-specific trends in head, pronotal and elytral shape may provide additional insight into the sexual selective pressures underlying morphological diversification.

2. Interspecific variation

Interspecific morphological variation was not explored in female *Colophon* as they could not be identified to species level and it remains for genetic methods or more detailed anatomical analyses to separate females according to species. This highlights their extreme morphological homogeneity, as is typical for most Coleopteran species (Kawano 2006; Switala 2013). Male *C. haughtoni* and male *C. kawaii* demonstrated significant morphological differences in the mandibles, heads, pronota and elytra (Table 2). and these species could be accurately classified using these structures. Size (LnCS) significantly affected shape variation in all structures (Figs. 4a-d) and made a large contribution to species separation in both PCAs and DFAs (Tables 1 and 3). *C. kawaii* were significantly larger than *C. haughtoni* for all structures. The results of this study showed species-specific morphological variation in *C. haughtoni* and *C. kawaii*, giving support to the hypothesis that these are distinct species that are able to hybridise.

2.1. Mandible

Coleopteran weapons are impressive not only for their size but also their diversity and male *Colophon* are no exception to this. *C. haughtoni* were shown to have a longer ventral process than *C. kawaii* and less distinct mandibular horns due to a flattened surface connecting the mandible apex to the dorsal process (Figs. 1, 3a). Although all male beetle weapons are used in male-male competition it is not yet clear why these organs are so diverse, given their common function (Emlen et al. 2005). Palmer (1978), Eberhard (1981) and Siva-Jothy (1987) suggest that the variation in Coleopteran weapon morphology mirrors species-specific differences in how they are utilised in combat; specialized knobs and spikes may provide leverage and friction specific to particular fighting tactics (Emlen et al. 2005). Male *C. haughtoni* have been observed to use their mandibles to attack an opponent's gula (throat) or leg (T.E., pers. obs.). However further observations and statistical analyses for both species are necessary to confirm whether the divergent mandible morphology of *C. haughtoni* and *C. kawaii* is related to differing combat behaviour.

2.2. Head

Compared to *C. haughtoni*, *C. kawaii* heads were bigger and broader. As previously discussed, changes in mandible size, form and function are shown to produce changes in the head morphology of male and female *C. metallifer* stag beetles (Goyens et al. 2014) and also between different ant species (Paul and Gronenberg 1999). The observed divergence in *Colophon* head shape may similarly result from the larger, differentially shaped mandibles of *C. kawaii* compared to *C. haughtoni*.

2.3. Pronotum

The larger pronotum in *C. kawaii* may be due to the increase in overall body size but no explanation could be found for the more protrusive shoulders observed in this

species (Fig. 3c). Some studies find that morphological variations are correlated with ecological factors (Forsythe 1991; Barton et al. 2011). For example, mandible length is correlated with prey size in tiger beetles (Coleoptera: Carabidae) (Pearson and Mury 1979; Ganeshaiah and Belavadi 1986) and many insect species show significant trends in body size with increasing altitude (Chown and Klok 2003). There is insufficient information on *Colophon* to make assumptions regarding the ecological drivers of their morphological diversification but quantifying this diversity is the first step in elucidating the selective pressures underlying their speciation.

3. Accuracy of geometric morphometrics

This study has confirmed both the power and the shortcomings of geometric morphometrics for *Colophon* identification. Intraspecific variation could not be analysed in females without *a priori* species identification but the DFA distinguished them from males with 85-100% accuracy for all structures. Males could be identified to species level with 100% accuracy based on their mandibles but also with high percentages of accuracy (72.7-90.6%) using just heads and pronota. The remote distribution and elusive nature of *Colophon* poses a barrier to scientific research (Switala et al. 2015) and often only a few incomplete fragments are the only evidence to be found in the field (Endrödy-Younga 1986; T.E., pers. obs.). Scientists are heavily reliant on mandible morphology for species and sex identification (Switala 2013) but due to *Colophon*'s relatively small size the head and mandibles are often missing (T.E., pers. obs.). Whilst genetic methods are most popular for insect species identification (Behura 2006) the provincial laws protecting *Colophon* beetles (CITES 2015) also hinder the acquisition of fresh specimens for genetic analysis. In addition, species including *C. haughtoni* and *kawaii* inhabit private land and approval from landowners to remove these endangered and valuable beetles

may be problematic (T.E., pers. obs.). To aid species identification in the field Switala (2013) attempted to determine diagnostic larval characters for *Colophon*. However as with many scarabaeoids, interspecific differences were too small for this purpose. Geometric morphometrics could provide a reliable, fast and cost effective technique for *Colophon* identification which could be particularly valuable when attempting to map species distributions or locate new populations.

Conclusion

Prior to this study interspecific and intersexual shape variations were observed but not yet quantified in *Colophon* mandibles and the morphological variation of the head, pronotum or elytra was not considered for the genus. Only *C. haughtoni* and *C. kawaii* were analysed in this study but we showed that geometric morphometrics can be a powerful technique for exploring interspecific and intersexual variation in any *Colophon* species. These methods allowed the detection and statistical analysis of subtle shape variations that were previously unknown and identified body structures other than the mandibles that could be used for species and sex discrimination. Sexual dimorphism is evident in the mandibles of all *Colophon* species but we also showed significant dimorphisms in the head, pronotum and elytra of *C. haughtoni* and *C. kawaii*. The morphological distinction of heterospecific male beetles gave support to the hypothesis that *kawaii* is a valid species.

Our results provided novel insights into the interspecific and intersexual shape diversity of *C. haughtoni* and *C. kawaii* and identified additional avenues for study. Further research on sexual dimorphism could give insight into the reproductive biology of the genus, which is currently unknown. Also, it is important to understand how sexual selection has shaped *Colophon* evolution (e.g. male weaponization and

527 hypertrophy) and whether interspecific differences in male beetles could reflect
528 ecological adaptations such as altitudinal size variation. Finally, geometric
529 morphometrics could help to elucidate *Colophon* phylogeny and create a taxonomic
530 key for the entire genus.

531 Ultimately, geometric morphometrics could aid *Colophon* conservation by facilitating
532 accurate species identification, thereby enabling taxon-specific, targeted
533 conservation strategies. Thereafter geometric morphometrics could give insight into
534 the reproductive biology, ecology and distribution of this rare and endangered group
535 of beetles.

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Tables:

Table 1. Size variation and allometry in male *C. haughtoni*, male *C. kawaii* and female *Colophon* (species unknown). ANOVAs tested for significant differences in the natural logarithm of centroid size (LnCS) between beetle groups. Shape variables were regressed on LnCS to test for allometry in each structure. % expl. var. is the variation in each dataset explained by size. Goodall's *F*-test analyses the significance of the regression.

	ANOVA			% expl. var.	Goodall's <i>F</i> -test		
	<i>F</i> -ratio	d.f.	<i>p</i> -value		<i>F</i> -ratio	d.f.	<i>p</i> -value
Mandible	30.92	1, 49	<0.0001	15.80%	9.25	6, 294	<0.0001
Head	241.9	2, 36.64	<0.0001	41.96%	63.07	8, 696	<0.0001
Pronotum	145.28	2, 27.14	<0.0001	42.68%	49.94	8, 536	<0.0001
Elytron	31.42	2, 60	<0.0001	20.21%	15.45	4, 244	<0.0001

Table 2. Results of non-parametric MANOVAs (9999 permutations) and pairwise comparisons performed on principal component scores from the mandibles, heads, pronota and elytra of male *Colophon haughtoni*, male *C. kawaii* and female *Colophon* (species unknown).

	MANOVA		Pairwise comparisons		
	<i>F</i>	<i>p</i>	groups	<i>F</i>	<i>p</i>
Mandible	65.17	0.001	<i>hau</i> x <i>kaw</i>	65.17	0.001
Head	70.09	0.0001	<i>hau</i> x <i>kaw</i>	16.32	0.0001
			<i>hau</i> x <i>fem</i>	110.3	0.0001
			<i>kaw</i> x <i>fem</i>	93.45	0.0001
Pronotum	38.33	0.0001	<i>hau</i> x <i>kaw</i>	9.392	0.0001
			<i>hau</i> x <i>fem</i>	53.83	0.0001
			<i>kaw</i> x <i>fem</i>	42.76	0.0001
Elytron	14.38	0.0001	<i>hau</i> x <i>kaw</i>	3.629	0.02
			<i>hau</i> x <i>fem</i>	19.22	0.0001
			<i>kaw</i> x <i>fem</i>	21.35	0.0001

Table 3. Canonical discriminant coefficients and loadings for the discriminant function analyses applied to body structures of *Colophon* beetles. The shape (principal component (PC) scores) and size (natural logarithm of centroid size (LnCS)) of body structures were used to distinguish between male *C. haughtoni*, male *C. kawaii* and female *Colophon* (species unknown). The standardized coefficient indicates the contribution of each size/shape variable to the discriminant function(s) derived from the analysis. Loading represents the correlation between the discriminant function(s) and an independent variable (beetle group).

	Standardized Coefficient		Loading	
	Function 1	Function 2	Function 1	Function 2
(A) Mandible*				
LnCS	-0.634		-0.251	
PC1	1.342		0.545	
PC2	-0.490		-0.073	
PC3	0.603		0.088	
PC4	-0.604		-0.034	
(B) Head				
PC1	1.133	0.125	0.785	-0.443
PC2	0.723	0.775	0.08	0.732
PC4	0.094	0.552	0.007	0.344
PC5	-0.307	-0.003	-0.021	-0.072
LnCS	-0.135	0.491	-0.336	0.607
(C) Pronotum				
LnCS	-0.494	0.164	-0.764	0.172
PC1	0.739	0.308	0.777	0.520
PC2	0.285	-0.588	0.152	-0.742
PC8	-0.137	0.586	-0.42	0.642
(D) Elytra				
LnCS	0.688	0.623	0.657	0.336
PC1	0.675	-0.814	0.593	-0.788
PC2	0.666	0.331	0.221	0.451

*Only one Discriminant Function was selected for the mandible dataset

Figure Legends:

Fig. 1. Adult *Colophon* beetles. (A) *Colophon haughtoni*. (B) Ventral photograph of *C. haughtoni* head showing (1) gena, (2) mandible base, (3) ventral process, (4) dorsal process, and (5) apex of the mandible. (C) Ventral view of *C. kawaii* head. Scale bars represent 4mm (A) and 2mm (B-C). Photographs by H.J. de Klerk.

Fig. 2. Landmarks used for geometric morphometric analysis of *Colophon* specimens: (A) male *C. haughtoni* mandible; (B) male *C. kawaii* mandible; (C) male *C. haughtoni* head; (D) female head; (E) pronotum; (F) elytron. Scale bars represent 2mm.

Fig. 3. Plots of the first two principal component (PC) scores obtained from principal component analyses on the shapes of four structures in *Colophon* beetles; (A) mandible, (B) head, (C) pronotum and (D) elytron. The analysis was carried out on male *C. haughtoni*, male *C. kawaii* and female *Colophon* of unknown species. The values in brackets for each axis represent the percentage of shape variance explained by each PC. Deformation grids show the shape change from the consensus to the extreme positive and negative of each PC axis.

Fig. 4. Box plots and deformation grids showing size variations and shape deformations in *Colophon* beetles: female *Colophon*, male *C. haughtoni* and male *C. kawaii*. Size was measured as natural log transformed centroid size (LnCS). Deformation grids show the shape changes related to size from the smallest to the largest individuals. Values in parentheses are the magnification applied to improve visualisation of shape deformations.